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Isolation and Characterization of Anti-SEB Peptides Using Magnetic Sorting and Bacterial Peptide Display Library Technology

by Joseph M. Pennington, Joshua M. Kogot, Deborah A. Sarkes, Paul M. Pellegrino, and Dimitra N. Stratis-Cullum

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14. ABSTRACT

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Peptide display libraries offer an alternative method to existing antibody development methods enabling rapid isolation of highly stable reagents for detection of new and emerging biological threats. Bacterial display libraries are used to isolate new peptide reagents within 1 week, which is simpler and timelier than using competing display library technology based on phage or yeast. Using magnetic sorting methods, we have isolated peptide reagents with high affinity and specificity to staphylococcal enterotoxin B (SEB), a suspected food pathogen. Flow cytometry methods were used for on-cell characterization and the binding affinity (K_d) of this new peptide reagent was determined to be 56 nm with minimal cross-reactivity to other proteins. These results demonstrated that magnetic sorting for new reagents using bacterial display libraries is a rapid and effective method and has the potential for current and new and emerging food pathogen targets.

Keywords: peptide, SEB, synthetic recognition element, magnetic sorting, affinity reagent, bacterial display

1. INTRODUCTION

Traditionally, biosensors have utilized antibodies for capture reagents to sense bio-threat targets, effectively using the mechanism employed in our own immune systems. Antibodies are Y-shaped proteins that detect bacteria and other foreign objects using a "lock-and-key" mechanism to bind to specific regions called antigens signaling them for destruction. Immunology has capitalized on the body's ability to produce antibodies against toxins by exposing immunized hosts to a toxin and harvesting the antibodies it produces to be used in other applications[1]. Immunized hosts often produce monoclonal antibodies, which are high affinity, very selective proteins produced in B cells in the host's spleen. The high affinity and selectivity of monoclonal antibodies (mAbs) make these proteins the gold standard for recognition reagents in detection and sensing. After initial immunization, the spleen of the host animal is excised for isolating B cells, which will be combined with cancerous, immortalized B cells for in vitro production of monoclonal antibodies. Another method known as polyclonal serum also uses antibodies but produces multiple antibodies that bind to multiple antigen sites, sometimes non-specifically. While both methods produce antibodies capable for use in biosensors, they are relatively fragile and present logistical hurdles for use in the field. Furthermore, there is a long development time for antibodies ranging from weeks to months, which limits the swiftness to respond to an emerging, unknown biological threat. As an alternative to antibody technology, synthetic peptide alternatives, based on bacterial display, phage display, and yeast display libraries are being explored. The development time of these reagents can be completed in weeks or even days, 3-5 days, with bacterial display technologies[2-5].

Synthetic peptide alternatives can be selected using a combinatorial strategy that employs a peptide display library that uses cellular machinery to generate a library of randomly generated peptide sequences that have been displayed on the surface of the cell (typically yeast, phage, or bacteria). Phage display libraries have been used to sort against a number of targets, including staphylococcal enterotoxin B (SEB), but have produced variable results [6, 7]. A cell surface display method has been developed for use on the exterior membrane of bacteria by expressing a protein with both a randomized peptide on the N-termini and an expression control peptide on the C-termini [8]. Bacterial surface displays have been widely used due to the diversity of peptides able to be displayed (usually 10^9-10^{11} discrete peptide mutants)[8, 9]. To select the peptides with the strongest affinity, a magnetic sorting technique can be used by conjugating the toxin of interest directly to a magnetic bead. Therefore, only those displayed peptides that bind to the toxin are selected for and separated from the rest of the library. By decreasing the amount of toxin through multiple rounds of sorting, the strongest affinity binders can be selected [3].

Following each magnetic sort, the enriched libraries as well as individual clones can be characterized by flow cytometry to determine the relative binding affinity to the original target. Flow cytometry is a technique that can rapidly monitor and quantify cells based on relative size, complexity, and fluorescence. For surface displayed peptides, a target of interest can be conjugated directly to a fluorescent dye enabling a direct correlation between target bound (fluorescent) and cells with no binding (no fluorescent signal). Whole library as well as single clone characterization can be performed by establishing population gates to quickly view (i.e., gate) the changes in population from cells that do not fluoresce from those that do exhibit positive results.

Staphylococcal enterotoxin B (SEB) is one of the seven enterotoxins of *Staphyloccocus aureus* and is one of the most well understood and well studied staph enterotoxins [10]. SEB is a 29-kDa superantigen, so-named because of the extreme immune system effects, including toxic shock syndrome[10]. SEB is thermally stable retaining 50% of the biologicial activity at 100°C exposure for 5 mins and is stable across a pH 4-10[11]. Detection of the staphyloccocal enterotoxins in food is critical since food poisoning is often caused by enteric exposure of these enterotoxins, resulting in emesis and diarrhea[12]. The inhalation ED50, the dose to incapacitate 50% of the population, is 0.0004 μg/kg and the LD50, lethal dose to 50% of the population, is 0.02 μg/kg; the low effective quantities makes the SEB toxin a potential biothreat agent[10]. The potential threat of SEB in food safety or biowarfare threat necessitates a detection reagent that is stable across many temperatures and pH, is high affinity, and is highly selective. Using a bacterial display library expressing a random 15 amino acid peptide on the outer membrane, we show that it is possible to produce a peptide reagent with high affinity and high specificity for SEB.

2. METHODS

2.1 SEB sample

SEB (Sigma-Aldrich) was supplied as a lyophilized powder from Sigma-Aldrich. A 1 mg sample was dissolved in 0.5 ml sterile, deionized water and split into two equal samples, one for dye-labeling for flow cytometry analysis and one for magnetic bead labeling for sorting. The SEB was dye-labeled for flow cytometry analysis using DyLight488 (Thermo Scientific) according to the manufacturer's instructions. Unreacted dye was removed using a 5,000 nominal molecular weight limit (NMWL) dialysis membrane. The magnetic bead-SEB conjugate was prepared using Magnabind Carboxyl Derivatized Beads (Thermo Scientific) according to the manufacturer's instructions. Briefly, the SEB was conjugated through surface exposed lysine residues, primary amine groups, to the carboxyl surface using standard EDC-NHSS conjugation. After the coupling reaction was complete, the bead-bound and unbound SEB were purified using a

	Round 1	Round 2	Round 3	Round 4
Cells for Sorting	10X the library diversity (2×10^{11} for eCPX library)	5 – 10 X the depleted library diversity (at least 1×10^8 cells for bacterial display)	5 – 10 X the depleted library diversity (at least 1×10^8 cells for bacterial display)	5 – 10 X the depleted library diversity (at least 1×10^8 cells for bacterial display)
Magnetic Beads	100 μl	15 μl	8 μl	4 μl
SEB Concentration	600 nM	300 nM	150 nM	75 nM
Sample Volume **Mixing Buffer Vol.** **Wash Buffer Vol.**	2 mL 3 mL 8 mL	500 μL 800 μL 8 mL	500 μL 800 μL 8 mL	500 μL 800 μL 8 mL

Table 1: Typical parameters used in magnetic sorting of bacterial display libraries using the MMS instrument, microfluidic magnetic sorter (Cynvenio Biosystems, Inc).

magnet, and the concentration of SEB on the magnetic beads was determined by measuring the A_{280} before and after coupling.

2.1 Magnetic sorting

A bacterial display library (from frozen stock) was inoculated into 500 mL of Luria Broth supplemented with 25μg/ml Chloramphenicol (LB-Cm^{25}) and grown at 37°C with vigorous agitation (250 rpm). The library was grown to an optical density (OD_{600}) of 0.65 and induced with 0.04% L-Arabinose for 45 minutes. A 10-fold diversity over-sampling of 2 x 10^{11} cells were pelleted @ 3000xg for 20 minutes and resuspend in 1.5 mL phosphate buffered saline supplemented with 0.1% bovine serum albumin (PBSB) then transferred to a 2 mL microcentrifuge tube. The cells were combined to a 500 nM concentration of SEB conjugated magnetic beads and incubated for 60 minutes prior to sorting using a MMS sorter (Cynvenio Biosystems) according to previously described protocols [3]. Serial dilutions of the positive sort were plated onto LB Cm^{25} plates and incubated at 37ºC for 24 hrs. The remainder of the sorted cells were added to 5 ml of fresh LB Cm^{25} and incubated overnight with shaking at 37ºC. After a 24 hr growth the library was passaged into fresh media to repeat the process for subsequent sorting with more stringent antigen concentrations outlined in Table 1. A total of four rounds of sorting were completed with SEB concentrations of 600 nM, 300 nM, 150 nM, and 75 nM for rounds 1-4 respectively.

2.2 Clone characterization by flow cytometry

Single clones were selected, at random, from overnight growth plates after a round of sorting and grown in LB Cm^{25}, followed by a short induction with 0.04% L-Arabinose. Following induction, 5 μL of bacterial cells are added to 1.5 mL microcentrifuge tubes containing either 25 μL of PBS + 0.5% BSA (negative control), 150 nM Ypet Mona (positive control)[13], or 150 nM SEB-488. Cells were incubated for 45 minutes at 4ºC to allow binding to occur. The labeled cells were then centrifuged at 5,000xg for 5 minutes and resuspended in 1 mL FacsFlow© (BD Biosciences) before being analyzed on the BD FACSCanto II flow cytometer (BD Biosciences). Populations were gated off the negative control, providing a relative percent binding for each sample. The FITC (530/30 nm) and PE (585/42 nm)

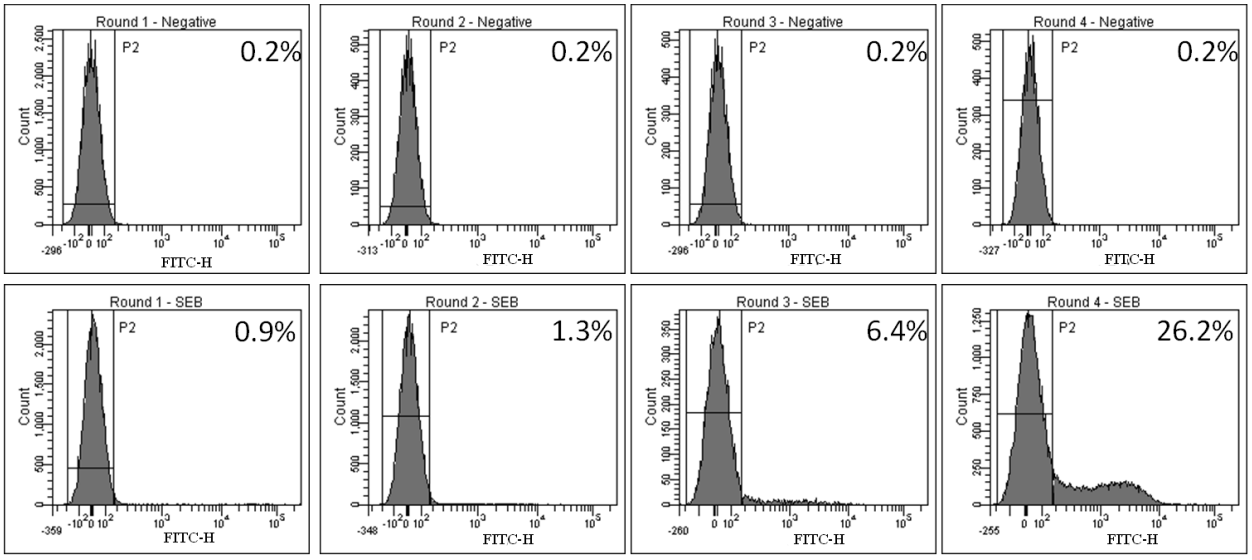

Figure 1: Characterization of SEB library binding following magnetic sorting for rounds 1-4. The top panel of data is the negative control samples for each round of sorting, and the bottom panel is the positive samples, indicated by the percent SEB binding as determined by flow cytometry. Each round of sorting, both positive and negative, is labeled successively. As each round of sorting is complete, an enrichment of SEB binders occurs, as noted by the increased percent SEB binding in the FITC channel (530/30 nm) measured by total fluorescence counts. The measured SEB binding increased from 0.9% in round 1, to 1.3% in round 2, 6.4% in round 3, and 26.2% in round 4. The negative control, native bacterial fluorescence in the FITC channel, was measured as 0.2%

channels were used to detect the fluorescent dyes after excitation using a blue laser (488 nm solid state, 20 mW laser).

PMTV parameters for forward scattering (FSC), side scattering (SSC), FITC, and PE channels were set to 537, 381, 436, and 411, respectively. Thresholds were added to the FSC and SSC channels (700,700) to remove background debris, allowing only bacterial cells to be recorded.

The binding dissociation constant (K_D) was determined by plotting the percent binding from flow cytometry analysis versus the concentration of SEB and fit using a sigmoid function with IGOR Pro (Wavemetrics, Inc). To determine specificity, the clones were analyzed against Strepavidin-R-phycoerythrin (SAPE), Neutravidin-R-phycoerythrin (NAPE), Dylight-488 conjugated Protective Antigen (PA488), and Dylight-488 conjugated anti-Hemagglutinin (HA488) at 1000 nM by flow cytometry characterization.

3. RESULTS and DISCUSSION

3.1 Magnetic enrichment of SEB sorting

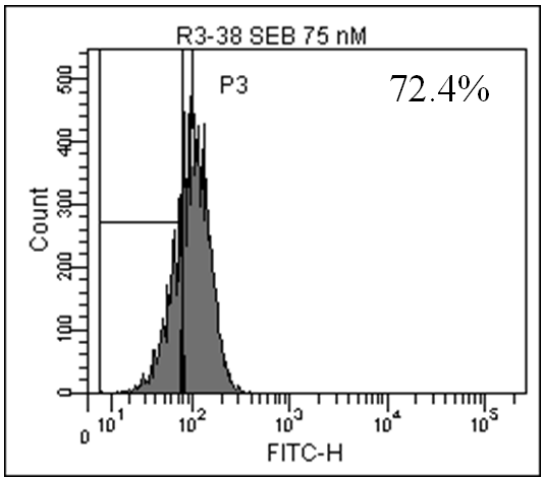

Figure 3: Flow cytometry, on-cell characterization of single clones of SEB binders. The SEB binding of one of the isolated clones, R3-38 is shown. This clone showed 72.4% SEB binding, above background, when measured with a 75nM SEB sample.

were not pursued.

3.2 Single clone characterization by flow cytometry

Single clones are typically measured once the whole library shows increased enrinchment after each round of sorting (Fig. 1), with single clones often measured in round 3. The single clones each contribute to the overall SEB binding noted in Round 4 in Fig. 1 (26.2% after Rd. 4). In Figure 2, a binding clone of the total 75 clones tested after 3 rounds of sorting is shown. The clone designated as R3-38 was analyzed by flow cytometry to determine the overall SEB binding at 75 nM SEB. Of the clones characterized, only 3 clones in the analysis of round 3 clones showed measurable SEB binding (data not shown), which suggests that a fourth round of sorting

Magnetic sorting enables the selection of high affinity populations in bacterial display library through a concurrent positive population enrichment and negative selection depletion. The population enrichment can be tracked using flow cytometry and a dye-labeled target, noting the increase in overall binding population at each round of magnetic sorting. In Figure 1, the enrichment of SEB binders within each library for rounds 1 through 4 were measured to be 0.9%, 1.3%, 6.4%, and 26.2% respectively. This clearly shows an enrichment of surface displayed peptides that bind SEB when compared to each of the negative control samples, inherent cell fluorescence, was measured as 0.2% in each sorting round (Fig.1). Round 4 showed the greatest increase in library enrichment after the 75 nM selection due to the increased enrichment of higher affinity SEB binders and the depletion of cells that did not bind SEB at 75nM. Single clones were isolated and characterized after both rounds 3 and 4. Sequence analysis of clones after four rounds of sorting resulted in identifying a consensus sequence and multiple copies of the same sequences, which indicates that the library has been enriched with SEB binders and further rounds

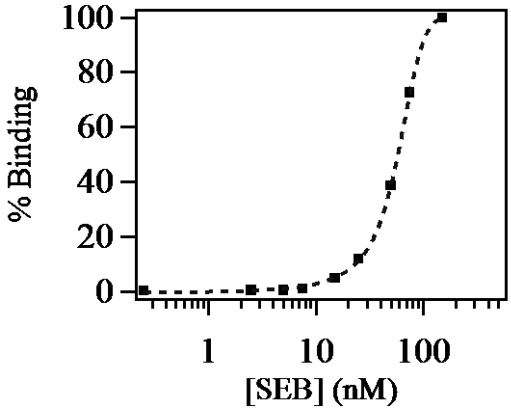

Figure 2: Binding dissociation analysis, on cell, for R3-38. The dissociation was determined by doing a SEB concentration dependent analysis by flow cytometry and plotting the percent binding at each SEB concentration measured. The curve as fit to a sigmoidal function and the K_D was determined to be 56.2 nM.

is necessary to further deplete the library of poor SEB binders. Alternatively, a greater statistical sampling of single clones in round 3 (more than the 75 tested) would likely result in a greater number of high affinity SEB binders in the round 3 population.

To determine the binding affinity (K_d), a FACS analysis was performed as a function of target concentration. From these data, the binding affinity of the R3-38 clone was measured, on cell, by measuring the total fluorescence of SEB-488 in a concentration dependent analysis (Fig. 3). The affinity for R3-38 was calculated to be 56.2 nM after fitting the concentration dependent binding of SEB to the R3-38 clone to a Sigmoid curve using Igor Pro. Moreover, the expected binding affinity is 150 nM or less since 150 nM of SEB is used for sorting in round 3 (Fig. 1).

3.3 On-cell specificity analysis of anti-SEB peptides

The overall specificity of the anti-SEB peptides was measured on-cell by determining the total percent cross-reacting protein binding as a function of concentration (Fig. 4). The cross-reacting proteins were selected based on proteins often used in downstream assay development, such as neutravidin (NAPE) and streptavidin (SAPE) for biotin binding, or other protein targets often assayed as potential biothreat agents, protective antigen (PA) from *B. anthracis* and hemagluttinin (HA) from influenza virus. In Figure 4, shows characterization of the anti-SEB peptide clone designated as R3-38 exhibited very good specificity when measured against 1000nM solutions of SAPE, NAPE, PA, and HA. The greatest overall cross-reactivity for R3-38 was found with the 1000 nM NAPE sample, approximately 10% binding. The R3-38 had similar cross-reactivity with the SAPE at 1000 nM, under 10% binding to SAPE, and had the lowest cross-reactivity binding with PA, approximately 5%, and the HA approximately 3%. It is important to note, the R3-38 binding to SEB was analyzed using 75 nM SEB-488, which is over a full order of magnitude less SEB-488 protein than any cross-reacting protein.

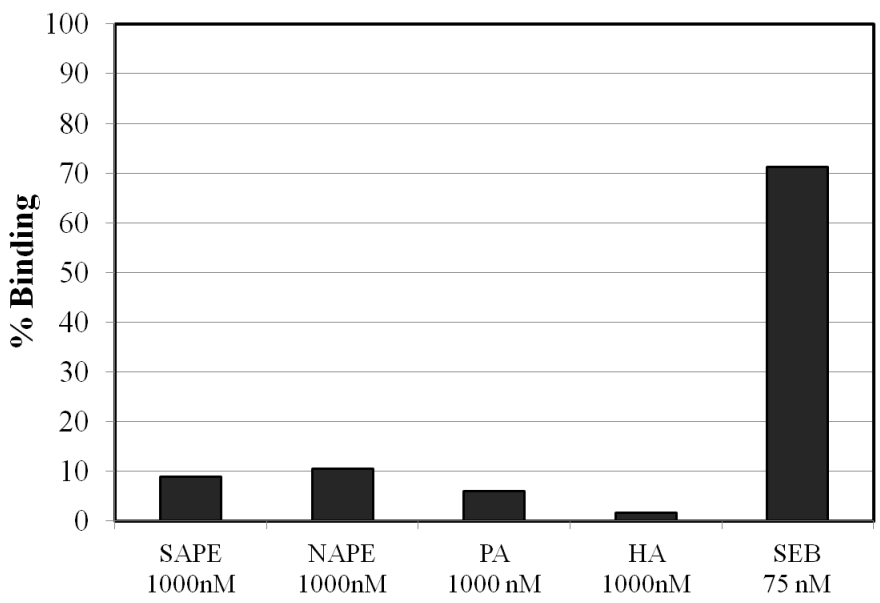

Figure 4: Specificity analysis of bacterial display clones using a cross reactivity panel of 4 proteins: SAPE, NAPE, PA, and HA. The R3-38 clone was incubated with 1000 nM of each of the potential cross-reacting proteins. The overall specificity is plotted as a percent binding for each clone with SEB binding at 75 nM provided as a reference.

5. CONCLUSION

Three or four rounds of magnetic sorting using an outer-membrane surface display bacterial library produced highly specific, high affinity peptide candidates for molecular recognition of SEB. Flow cytometry data detailing the enrichment over the negative control samples is noted in each round of sorting. The anti-SEB peptide binders analyzed on-cell showed high affinity for SEB, as evidence by the fluorescence saturation in flow cytometry when measured at 75 nM SEB-488. As expected, the SEB-488 fluorescence signal from flow cytometry analysis for the R3-38 (round 3 sorting) was not as large as the round 4 binders, >99%, when measured at 75nM SEB-488 since the round 3 clones were sorted at a higher concentration (150 nM SEB). Besides having the highest SEB-488 binding, the round 4 sorted clones also exhibited the greatest specificity to PA, NAPE, SAPE, and HA. Further analysis off-cell with these peptide reagents will infer the affinity and specificity compared to known SEB binders selected from phage display. Finally, correlations between on-cell and off-cell behavior will be inferred after anlaysis of the peptide reagents off-cell using standard immunoassay techniques including surface plasmon resonance (SPR), affinity capillary electrophoresis (ACE), and enzyme-linked immunosorbent assays (ELISA).

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